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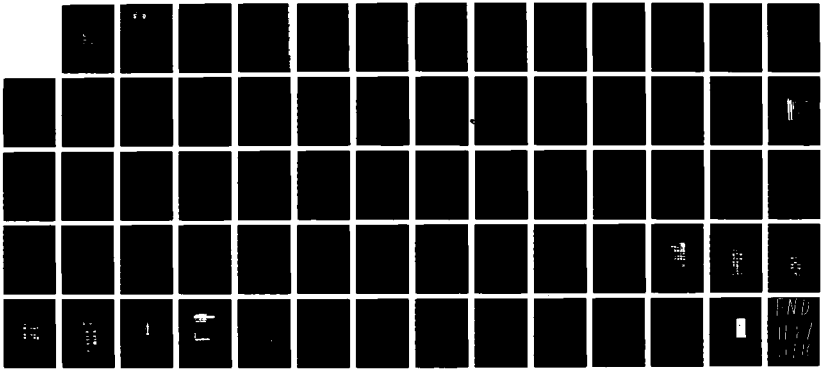

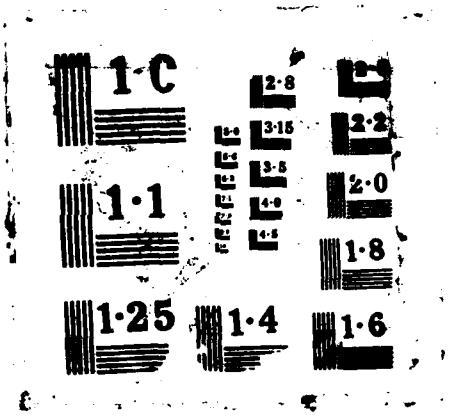
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The purpose of obtaining the gene of Adenosinediphosphoribosyl Transferase (ADPRT) is: 1) the complete amino acid sequence of this large protein is best determined from the DNA sequence of the gene, 2) isolation of the gene provides gene probes that permit location and quantitation of the gene within genomic DNA, and 3) a variety of biological experiments at the cellular level requires specific gene probes. The progress we made in two years is based on the success of a new method of isolation of the enzyme (I), determination of its peptide structure (II) and application of synthetic DNA probes (III) derived from amino acid sequences, resulting in the isolation of the largest DNA-insert from a gt 11 cDNA library, 5-6 kb, that contains the entire sequence for the ADPRT gene. Since the rest of isolation depends on fairly routine techniques (sequencing, hybrid-arrested translation, etc. methods) it is predictable that the gene from bovine origin is going to be identified within the third year of this specific project.

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PROGRESS REPORT

Molecular Cloning of Adenosinediphosphoribosyl Transferase

Period of September 1, 1986 - August 31, 1987

Submitted by: Professor Ernest Kun
Principal Investigator
University of California, San Francisco
Department of Pharmacology and the
Cardiovascular Research Institute

September 8, 1987

AFOSR - 85 -0377

PROGRESS REPORT

Molecular Cloning of Adenosinediphosphoribosyl Transferase

Period of September 1, 1986 - August 31, 1987

Submitted by: Professor Ernest Kun
Principal Investigator
University of California, San Francisco
Department of Pharmacology and the
Cardiovascular Research Institute

September 8, 1987

AFOSR -85 - 0377

Progress Report: Molecular Cloning of ADPRT

Period:September 1, 1986 - August 31, 1987

Ernest Kun, Principal Investigator

Summary Statment: The purpose of obtaining the gene of Adenosinediphosphoribosyl Transferase (ADPRT) is at least three-fold. First: the complete amino acid sequence of this large protein is best determined from the DNA sequence of the gene. Second: isolation of the gene provides gene probes that permit location and quantitation of the gene within genomic DNA. Third: a variety of biological experiments at the cellular level requires specific gene probes. Since the time this original application was submitted (end of 1984) a large scale competition for this problem began worldwide, and presently, according to reports presented at the Symposium at Fort Worth (Texas), seven laboratories are competing. The progress we made in two years is based on the success of a new method of isolation of the enzyme (I), determination of its peptide structure (II) and application of synthetic DNA probes (III) derived from amino acid sequences, resulting in the isolation of the largest DNA-insert from a gt 11 cDNA library, 5-6 kb, that contains the entire sequence for the ADPRT gene, in contrast to results of others, that report 1.5-2 kb insert DNA's. Since the rest of isolation depends on fairly routine techniques (sequencing, hybrid-arrested translation, etc. methods) it is predictable that the gene from bovine origin is going to be identified within the third year of this specific project.

It shoud be noted that partial support of this work is also due to the second AFOSR grant (86 - 0064), thus the two projects are cooperative.

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This report is submitted by Prof. E. Kun, UCSF, September 8, 1987

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*note: Transferase or Polymerase are interchangable terms.

I

Isolation of Adenosinediphosphoribosyl Transferase by Precipitation with Reactive Red 120 Combined with Affinity Chromatography[1]

[4]Abbreviations used: ELISA, enzyme-linked immunoadsorbent assay; SDS,sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AP-Sepharose, O-[[3-[(3-amino-propyl)amino]propyl]-guanyl]-Sepharose; AH-Sepharose 4B,O-[(6-aminohexyl)guanyl]-Sepharose 4B; 3-aminobenzamide-Sepharose 4B, O-[14-[3-(aminocarbonyl)phenyl]-1-imino-10,13-dioxo-2,9,14-triazatetradecyl]-Sepharose 4B.

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Abstract

The DNA-associating enzyme, adenosinediphosphoribosyl transferase, has been isolated from calf thymus by selective precipitation with a solution of dihydroxy-Reactive Red 120, followed by extraction of the enzyme from the precipitate with 2 M KCl and an on-line train of three successive column chromatographic steps, including a final 3-aminobenzamide-Sepharose-4B affinity chromatography. The method yields 8-9 mg of more than 95% homogeneous enzyme protein per kg starting material and requires about 3 working days. This dye precipitation method is distinct from affinity precipitation, since it involves the binding of the dye to both nonspecific sites and the substrate- and DNA-site of the transferase as indicated by enzyme inhibition by dihydroxy Reactive Red 120 at both enzyme sites.

Nuclei of eukaryotic cells above the evolutionary stage of genus saccharomyces (1) contain a specific DNA-binding protein that catalyzes the transfer of the ADP-ribosyl moiety of NAD^+ to proteins (for reviews see 2, 3). This enzyme, known as adenosinediphosphoribosyl transferase (E.C.2.4.2.30), also catalyzes the successive transfer of ADP-ribose to protein-ADP-ribose chain initiator adducts (4), generating homopolymers of ADP-ribose that possess a specific helical structure (5, 6). Resolution of the biological function of the transferase at a molecular level depends on a detailed understanding of the molecular structure of the enzyme including the identification of the transferase gene and its regulation. Progress in this field is enhanced by efficient procedures for the isolation of the enzyme from a convenient source (e.g. calf thymus). Various methods have been published (7, 8, 9, 10) requiring laborious fractionation techniques. Recently, a relatively rapid method has been described (11) which is based on affinity chromatography, with admittedly low yields. An affinity column of Reactive Red-agarose has been previously used for the isolation of the transferase (10) but we experienced poor yields with this method.

We find that an aqueous solution of the dihydroxy form of Reactive Red 120 precipitates the same quantity of DNA-binding and other basic proteins, including the transferase, as is adsorbed by the Reactive Red agarose column (10). However the purification procedure based on dihydroxy Reactive Red 120 - protein precipitation is much faster than gradient elution from the dye-agarose column and the yields of the enzyme are significantly higher than reported

for other affinity column methods (10,11).

We report here a method for the isolation of the transferase from calf thymus employing a solution of the dihydroxy form of Reactive Red 120 (structure shown in Fig.1) as a precipitating reagent for the enzyme. Within two to three working days an enzyme protein of at least 95% homogeneity is obtained, and proteolytic degradation and denaturation of the enzyme is thus minimized. A preliminary note concerned with this work has been published (13).

MATERIALS AND METHODS

Frozen calf thymus was obtained from Roth Products, Inc. (Gwynedd, PA), hydroxylapatite (BioGel HTP) from BioRad (Richmond, CA), [^{32}P]NAD from New England Nuclear Co.(Boston, MA), histones type IIA (H-9250), anti-rabbit IgG coupled to peroxidase (A-8275), and Reactive Red 120 (R-0378) from Sigma Co.(St.Louis, MO). All other reagents were of analytical grade. Anti-transferase antiserum was raised in rabbits as described for anti-poly(ADP-ribose) (14) and its titer was above 10,000 as tested by the ELISA assay. The antigen was a transferase preparation of 95-98% purity (7) as determined by SDS-PAGE, containing traces of polypeptides (60 and 40 kDa) that were proteolytic degradation products of the native enzyme (120 kDa).

3-Amino-benzamide-Sepharose-4B was prepared by a procedure similar to that described by Burtscher et al. (11), the AP-Sepharose matrix was replaced by AH-Sepharose 4B (Pharmacia), resulting

in a 1,6-hexanediamine linker on the resin. It was calculated from the known amino group content of AH-Sepharose-4B that on the basis of ΔA_{250} the benzamide substitution was nearly quantitative, yielding a benzamide content of the affinity resin of 5 μmol/ml in the hydrated gel.

Preparation of the dihydroxy Reactive Red 120 dye from the chloride form (see Fig.1). A batch of 4.4 g of the dye was dissolved in 100 ml of 0.1 M aqueous Na_2CO_3 , followed by the addition of 1 ml of pyridine. The solution was kept at 70°C for 24 hours, cooled to room temperature, and 400 ml of absolute ethanol was added dropwise from a separatory funnel during 5 minutes with stirring. The fine red precipitate formed during continuous stirring for 1 hour was collected by centrifugation, washed successively with ethanol and diethyl ether, then dried in vacuo. The yield was 3.4 g. Whereas the Reactive Red 120 in the chloride form reacted covalently with Whatman No.1 filter paper, as tested by soaking the paper overnight at 70°C in a solution of the dye (2 mg/ml) in 0.5 M Na_2CO_3 , the dihydroxy form (Fig. 1) gives no permanent staining reaction and the dye can be washed out of the filter paper with aqueous dimethylsulfoxide. This technique tests for the completion of the replacement of Cl by OH in the dye molecule. The dihydroxy Reactive Red 120 preparation gave one major spot with an R_f of 0.3, whereas the chloride form of the dye migrated with an R_f of 0.6 on silica gel TLC plates developed with ethyl acetate: butane-2-ol: propane-1-ol:H_2O = 10:20:35:35 (v/v). The spectrum of the product was almost identical with that of the starting dye ($\varepsilon_{530}=4.2 \times 10^4$).

Buffers:

Buffer I: Tris(base) 50 mM; EDTA(Na_2) 10 mM; NaN_3 1 mM; $NaHSO_3$ 50 mM; NaCl 0.3 M; 2-mercaptoethanol 10 mM; the pH was adjusted to 7.4 with 1 N NaOH.

Buffer II: Tris(base) 50 mM; EDTA(Na_2) 1 mM, NaN_3 1 mM; $NaHSO_3$ 50 mM; 2-mercaptoethanol 10 mM; glycerol 10% (v/v); the pH was adjusted to 7.4 as in Buffer I.

Buffer III: Tris(base) 50 mM; EDTA(Na_2) 1 mM; NaN_3 1 mM; 2-mercaptoethanol 10 mM; KCl 2.0 M, glycerol 10% (v/v) and ethylene glycol 15% (v/v), pH 7.4 was adjusted with 1 N HCl.

Buffer IV: HEPES 25 mM; 2-mercaptoethanol 10 mM; NaCl 200 mM; the pH was adjusted to 7.4 with 0.1 M NaOH. Phenylmethyl sulfonyl fluoride (PMSF) was added to the buffers to a final concentration of 0.1 mM from an ethanolic stock solution (100 mM) just before starting the isolation procedure.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels according to Laemmli (15). Covalently bound ADP-ribose-protein adducts were visualized in acid-urea-SDS gels in which they are stable (17).

Electrophoretic transfer from SDS-PAGE gels onto nitrocellulose membranes was carried out in a water cooled BioRad transblot cell in 25 mM 3-(cyclohexylamino)-2-hydroxy-1-propane sulfonic acid buffer containing 20% (v/v) methanol, following a published method (16) for 1.5 hours at 100 V.

Transferase activity was assayed in a reaction system composed of: Tris-HCl 50 mM, pH 8.0; $MgCl_2$ 10 mM; dithiothreitol 2.5 mM; NAD^+ at a final concentration of 0.5 mM containing [^{32}P]NAD (0.3-1

kBq per nmol); coenzymic DNA (7,19) and calf thymus histones (5 µg and 10 µg respectively per 50 µl test volume) and aliquots of the enzyme preparation to be tested. Incubation in 7 x 10 mm tubes lasted 3 minutes at 25 °C and the reaction was terminated by the addition of 2 ml of 10% cold $HClO_4$. Protein-bound polymer radioactivity was assayed by filtration onto GF/C filter discs (Whatman) which were then counted in a liquid scintillation spectrometer (17). One unit of enzymatic activity was defined as that amount of enzyme synthesizing one nanomol of protein-bound ADP-ribose per minute.

RESULTS AND DISCUSSION

Isolation of the enzyme. The transferase is a relatively rare nuclear protein, the estimated enzyme/DNA ratio of cells in culture varying between 1:200 to 500 kb DNA (18). The enzyme is highly susceptible to an as yet unidentified protease; therefore an uninterrupted sequence of purification steps greatly enhances its yield.

a. Tissue homogenization and ammonium sulfate fractionation. The entire isolation procedure was carried out in a cold room (4-6 °C). One batch of 250 g of frozen calf thymus per preparation was crushed to pea-sized pieces in a wooden box with a metal rod and the tissue fragments rapidly transferred into 500 ml of Buffer I, then immediately homogenized for 60 seconds in a blendor, followed by filtration through 4 layers of cheesecloth. The retained tissue on the cheesecloth was re-extracted with another portion of 250 ml Buffer I by blending for 10 seconds and

filtered through 4 layers of cheesecloth. The filtrates were combined and centrifuged at 7000 x g in 250 ml bottles at 4 °C for 15 minutes. Lipids were removed by refiltration through cheesecloth and the filtrate saved for ammonium sulfate precipitation as described (7). The fraction that precipitated upon addition of 23.08 g of solid ammonium sulfate per 100 ml was removed by centrifugation. To the supernatant 26.32 g of solid ammonium sulfate was added per 100 ml. The resulting precipitate contained the transferase. The precipitate was dissolved in 100 ml of Buffer II and if required can be stored at -70 °C for several weeks without loss of activity.

b. Precipitation of the transferase with dihydroxy Reactive Red 120. The protein fraction (see above), containing some $(NH_4)_2SO_4$, was diluted with Buffer II until its conductivity at 0-4 °C was lowered to 9-10 mSiemens. The volume at this stage was usually increased four fold (e.g., to 400 ml), and insoluble debris was removed by centrifugation, leaving behind a clear protein solution. To this solution, one tenth volume (e.g. 40 ml) of a precooled aqueous solution of dihydroxy Reactive Red 120 (10 mg dye/ml) was admixed with gentle stirring, whereupon a deep red precipitate was formed during the following ten minutes. The sodium salt of the dye is water soluble, but the dye anion precipitates with certain proteins. The precipitate was collected by centrifugation and washed with 40 ml of Buffer II containing 0.25 M KCl. The 0.25 M KCl-containing buffer removes loosely bound (adsorbed) proteins and only traces of the enzyme, and it prevents the extraction of dihydroxy Reactive Red 120, which would dissolve in Buffer II alone. The proteins precipitated by the dye, representing about 10% of total proteins present, are then eluted from the dye by suspending the precipitate in 160

ml of Buffer III (containing 2 M KCl). The insoluble potassium salt of the dihydroxy Reactive Red 120 is then removed by centrifugation.

The precipitation of dihydroxy Reactive Red 120 by increasing concentrations of K^+ is shown in Fig.2.

c. On-line column chromatographic and dialysis procedures. Final purification of the transferase from the above (160 ml) protein solution is carried out by a train of on-line connected column chromatography and fiber filter dialysis steps. The advantage of this procedure is that more than 98% of the proteins containing no enzymatic activity are rapidly washed through the system, while the transferase-containing protein fraction is selectively retained on the 3-amino-benzamide-Sepharose-4B column corresponding to the purified enzyme (step 4, Table).

This operation was carried out as follows. Three successive portions (5 g each) of hydroxylapatite powder were stirred in the slightly pink protein extract of the dihydroxy Reactive Red protein precipitate (see above, 160 ml). This amount of hydroxylapatite quantitatively adsorbed the enzyme, i.e.enzyme activity was absent in the supernatant. The slurry was poured into a short, wide column (4x6 cm) and then washed with Buffer III until non-adsorbed proteins were removed. The transferase-containing fraction was eluted with Buffer III containing 60 mM potassium phosphate and corresponded to about 150 mg protein (i.e. 50% of proteins present in the 2 M KCl-containing extract of the dihydroxy Reactive Red protein precipitate).

The effluent of the hydroxylapatite column was routed through a UV-monitor (λ = 280 nm) and connected thereafter to a hollow-

fiber dialysis unit (Spectra-Por HF) with a mol.wt. cutoff of 9000, containing 88 fiber units (Spectrum Medical Industries, Los Angeles, CA) that dialyzes against Buffer IV. The dialyzed solution was subsequently passed through a 10 ml bed of DEAE cellulose column (equilibrated with Buffer IV) to adsorb the last traces of the dye, and the effluent from here was led to a 4 ml bed of 3-amino-benzamide-Sepharose 4B that retained the transferase. The flux of the entire system (flow rate 0.4 ml/minute) was maintained until all proteins were eluted from the hydroxylapatite column and more than 98% emerged from the benzamide column as protein fractions containing no enzymatic activity. The transferase that was bound to the benzamide column was stable at cold room temperature and chromatography may be interrupted at this step for 24 hours without loss of activity.

Recovery of the transferase from the affinity column was preceeded by washing the column with 10 bed volumes of Buffer II containing 0.2 M NaCl, and the enzyme was then quantitatively eluted with the same buffer containing also 1 mM 3-methoxybenzamide and was recovered in a fraction collector. The volume of pooled enzyme fractions was reduced in Centricon 30 micro concentrators until a protein concentration of 4-8 mg/ml was obtained, and the 3-methoxybenzamide removed by several loads of Buffer IV in the same filter system. The enzyme solution, supplemented with 20 mM 2-mercaptoethanol and 20% glycerol, was stored at -70 °C without loss of enzymatic activity for at least 6 months.

The active, or coenzymic, DNA, which is a fragment of DNA that copurifies with the transferase in the early steps and is essential for

activity (7, 19), was retained on the hydroxylapatite column and was eluted by a phosphate gradient as described (19). Stages of purification are summarized in the Table, representing a typical experiment.

We have determined the specificity of the enzyme purification method by comparing mono-ADP-ribosylated protein bands at the four purification stages (see Table). As we have shown previously, at nanomolar concentrations of NAD^+, the transferase is auto-mono-ADP-ribosylated and the modified enzyme molecule is readily identifiable by urea-SDS-PAGE (4, 17). Only one mono-ADP-ribosylated enzyme band (120 kDa) was detectable at all stages of purification, indicating the absence of activity of other mono-ADPribose transferring enzymes (2, 3) under these conditions. A comparison of Coomassie-stained protein bands and immunotransblots at various stages of enzyme purification is shown in Fig. 3. In lanes 1 to 4 Coomassie blue stained bands illustrate the progress of purification (see Table), whereas lanes 5 to 8 are replica immunotransblots. Since the peroxidase-coupled immunostaining method is at least 10 times more sensitive than the Coomassie staining, traces of degradation products of the transferase are visible even at the highest stage of purification (lane 8), especially if the gel is overloaded (as in lane 8).

Precipitation of the enzyme by the -OH form of Reactive Red 120 is reminiscent of the known affinity precipitation of pyridine nucleotide-dependent dehydrogenases by various dyes, acting as NAD^+ analogs (20,21,22). However, affinity precipitation exhibits certain features that are not in common with the procedure described in the present paper. Generally, affinity

precipitation involves a mixing of an oligomeric enzyme with a bifunctional ligand, composed of two ligand entities connected by a spacer (20), providing a network of enzyme protein and bisligands. Precipitation of dehydrogenases by NAD^+ analog dyes is dependent on the presence of substrate, and the precipitate is redissolved by a solution of pyridine nucleotides (20, 21, 22). In our system, the precipitant dye binds the transferase and the precipitate is not dissociated by NAD^+, only by high K^+ concentrations. The binding of proteins to dihydroxy Reactive Red 120 probably involves several mechanisms. As seen from the Table and from lane 3 of Fig.3, only 10% of proteins are precipitated by the dye, suggesting that basic, presumably DNA binding nuclear proteins are among the ones which form ionic complexes with the dye, that by its polysulfonic acid nature may displace DNA and precipitate these proteins. Dihydroxy Reactive Red 120 at micromolar concentrations, where no precipitation takes place, inhibits the enzyme. In a test system containing the transferase(13 μg/ml), coenzymic DNA varying from 16 to 160 μg/ml and NAD^+ varying from 8 to 200 μM, the dye at 2 μM concentration shows competitive inhibitory kinetics ($K_I \approx 5$ μM) with respect to DNA when DNA concentration is varied and NAD^+ concentration is kept constant (200 μM). On the other hand, when DNA concentration is kept constant (160 μg/ml) and NAD^+ is varied, the dye at 0.4 μM shows competition with respect to NAD^+ ($K_I \approx 0.5$ μM). Various forms of mixed type inhibitions can be observed at widely varying concentrations of the dye, as would be anticipated also from the close vicinity of the NAD^+ and DNA binding sites on the enzyme,

which was calculated to be 8.5 Å (24), and from apparently allosteric mechanisms (4). The mechanism of the obligatory coenzymic function of DNA, that binds tightly to the transferase (7, 19, 23), is as yet controversial. However, evidence was obtained indicating that certain deoxyribonucleotide sequences may represent catalytically active specific binding sites (23) for the enzyme on DNA. Extrapolating from present results it seems possible that other DNA-binding proteins (see lane 3 of Fig.3) may also be isolated by the dye-precipitation technique,followed by specific affinity columns, that can selectively retain various DNA-associating proteins. This approach, which we are pursuing, may widen the scope of the techniques reported here.

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Legends

Figure 1:

Structure of Reactive Red 120 (Procion Red HE-3B, ICI)(12). The commercially available chloride form (X= -Cl) is converted to the unreactive dihydroxy form (X= -OH), which is used here as a precipitating agent.

Figure 2:

The precipitation of the dihydroxy dye by KCl(abscissa); left ordinate = the concentration of the dye in solution; right ordinate = conductivity that is proportional to the concentration of KCl. To a series of test tubes containing 10 ml of Buffer II with increasing concentrations of KCl, 1 ml of 10 mg/ml aqueous dye was added, mixed and kept at 0 °C for 10 min, then centrifuged. The dye content of the supernatant was determined by photometry at λ =530 nm and the conductivity was measured in a precooled conductometer.

Figure 3:

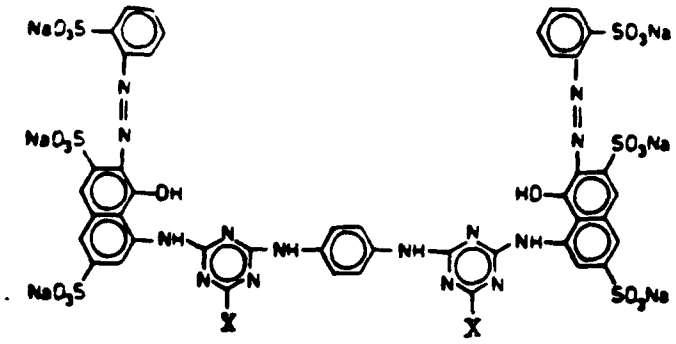
Comparison of protein bands and immunotransblots at four stages of purification. The quantities of protein applied to each slot in the gel were as follows: lane 1: 60 µg; lane 2: 50 µg; lane 3: 20 µg; lane 4: 5 µg of purification steps 1; 2; 3; and 4, respectively (see Table). Lanes 1 to 4 show protein bands (Coomassie stain), lanes 5 to 8 the corresponding immuno transblots.

Table

Typical purification of adenosinediphosphoribosyl transferase

Step	Volume (ml)	Total protein (mg)	Total units (nmol /min)	Units per mg
1. Crude Extract	750	21,250	22,000	1.0
2. $(NH_4)_2SO_4$-cut	100	3,500	21,000	6.0
3. Eluate from Red dye	160	350	9,500	27
4. Isolated Transferase	0.5	2.2	2,000	910

Fig 1.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NaO3S
SO3Na
X
X

Fig 2

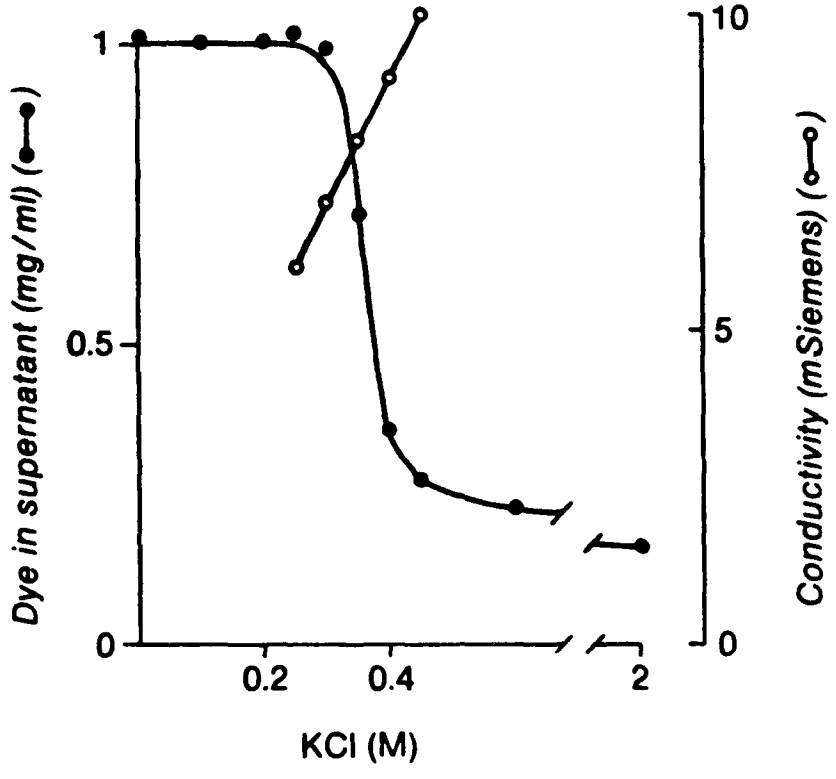
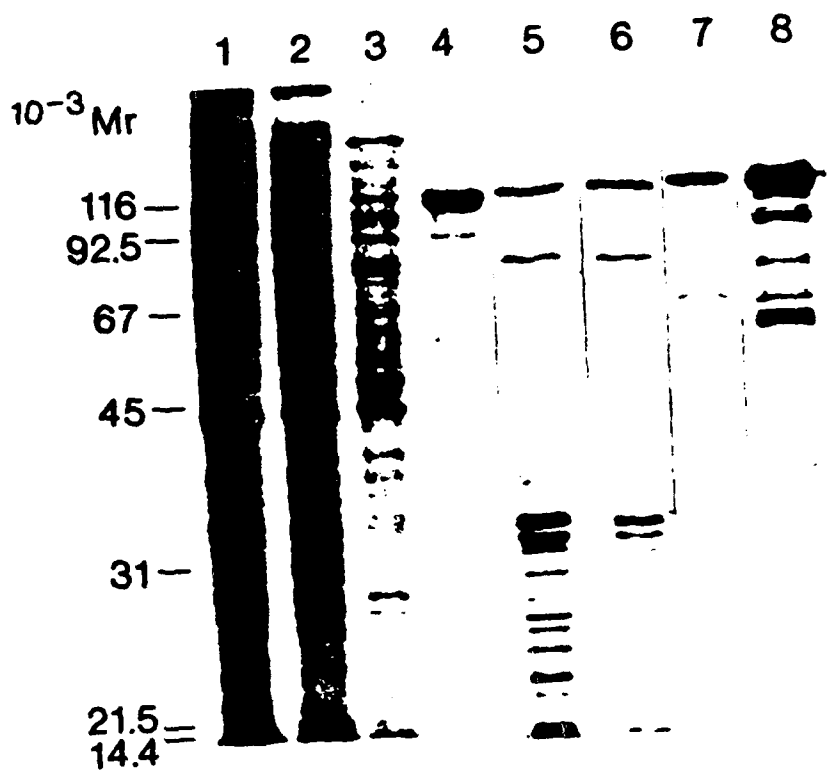

1
0.5
0
0.2
0.4
2
KCl (M)
Dye in supernatant (mg/ml) (●—●)
10
5
0
Conductivity (mSiemens) (○—○)

Fig 3



II

Polypeptide Components of Adenosine-diphosphoribosyl Transferase Obtained by Digestion with Plasmin†

Footnote to Table

[a] Values listed are mol %; cysteine is probably underestimated since -SH groups were not derivatized prior to hydrolysis. Tryptophan was not determined.

[1] Abbreviations: ADPRT, adenosine diphosphoribosyl transferase (E.C.2.4.2.30); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, Ca^{2+}- and Mg^{2+} free phosphate buffered saline; PMSF, phenylmethyl sulfonyl fluoride; PTH-, phenylthiohydantoinyl-.

Abstract: The adenosine diphosphoribosyl transferase enzyme (ADPRT) was hydrolyzed by plasmin to a few relatively stable large polypeptides that were characterized by amino acid composition, partial amino acid sequencing, affinity chromatography, antibody binding, and ADP-ribosylation. Proteolysis by plasmin inactivated ADPRT, and enzymatic activity depended exclusively on the intact enzyme molecule. The DNA-binding domain was split into two basic polypeptides, the one of M_r 29,000 contained the blocked amino terminus of the enzyme. The second basic polypeptide was actually a mixture of two of the same size (M_r 36,000). The latter were separated on an anion exchange column and sequenced to 51 amino acid residues, where they were found to differ by one lysyl residue at the N-terminal. The NAD^+-binding domain of the enzyme was present in the polypeptide of M_r 56,000, which consisted of a triplet, each polypeptide with a differing amino terminus. The M_r 56,000 polypeptide was further cleaved by plasmin to M_r 42,000 doublet fragments, both polypeptides posessing the same amino termini. The M_r 42,000 polypeptide was sequenced to 32 amino acid residues. All polypeptides were recognized by a polyclonal antibody raised against the intact enzyme. When the polypeptide fragments were enzymatically ADP-ribosylated, only the basic DNA-binding polypeptides served as ADP-ribose acceptors. By first auto-[^{32}P]ADP-ribosylating and then digesting ADPRT with plasmin, the acceptor sites were found to be distributed along the entire length of the molecule - i.e. all of the major polypeptides were labeled, most of the label being on the M_r 36,000 and 56,000

peptides. A reconstructed polypeptide model for the enzyme molecule is proposed that also specifies the N-terminal end of the polypeptide backbone and the location of DNA- and NAD^+ - binding domains.

Adenosinediphosphoribosyl transferase (ADPRT[1], poly(ADP-ribose)-polymerase E.C.2.4.2.30) is a DNA-associated nuclear enzyme which synthesizes protein-bound homopolymer of ADP-ribose utilizing NAD+ as a substrate. The enzyme itself is the main acceptor protein of ADP-ribosylation. The purified enzyme requires double-stranded DNA for activation (coenzymic DNA). It consists of a single large polypeptide chain of M_r 120,000; (reviews: Ueda & Hayaishi, 1985; Gaal & Pearson 1985). It is present in eukaryotes above the genus Saccharomyces (Scovassi et al., 1986). The polypeptide structure of this enzyme protein was first studied by Holtlund et al. (1983) and by Kameshita et al. (1984) and the latter group reported that limited chymotryptic digestion splits the enzyme into two polypeptides of M_r 66,000 and 54,000 respectively, and papain at 0 ℃ generates another pair of fragments (M_r 74,000 and 46,000). Distinct NAD^+- and DNA-binding domains of ADPRT were proposed and a peptide of M_r 22,000 was suggested as the only ADP-ribose acceptor domain (Kameshita, et al., 1984). Combining the peptides of M_r 74,000 and 46,000 in the presence of coenzymic DNA recovered about 20% of the original enzymatic activity (Kameshita et al., 1986), suggesting that interaction of both is needed for catalysis. Monoclonal antibodies raised against the enzyme recognized epitopes on the DNA- and the NAD^+-binding polypeptides but not on the supposedly automodifiable peptide M_r 22,000 (Lamarre et al., 1986).

Although the amino acid composition of polypeptides of ADPRT prepared by digestion with α-chymotrypsin and papain have been

reported, (Kameshita et al., 1986), amino acid sequences are as yet unavailable in the literature. The amino terminus of ADPRT is known to be blocked, but the nature of the blocking group has not been identified (Holtlund et al., 1981).

Limited proteolysis by suitable proteases would be expected to yield polypeptides which may provide further information regarding the structure of ADPRT. Kameshita et al. (1984) were able to present a gross picture of the primary structure by splitting the enzyme into two halves either with papain or with α-chymotrypsin In our experience, further proteolysis of ADPRT by α-chymotrypsin and papain yielded a progressive breakdown of polypeptides (not shown). In contrast to these, plasmin - a trypsin-like serine protease - produces four relatively stable polypeptides, three out of these accounting for the whole enzyme molecule. We exploit plasmin's fastidiousness (Robbins & Summaria, 1970) here to obtain a more refined picture of the structure of the ADPRT enzyme protein.

Experimental Procedures

Materials Reactive Red 120-agarose (R503), plasmin (P7911), and anti-rabbit IgG-peroxydase conjungate (A8275) were purchased from Sigma (St. Louis, MO.) and 3-methoxybenzamide from Pierce Chemicals (Rockford, IL.). For autoradiography, X-Omat AR (Kodak, Rochester, NY.) was employed. The Spherogel TSK 3000 SW (60 cm long) molecular size exclusion column was obtained from Beckman-Altex (Berkeley, CA.) and Mono-S HR 5/5 cation exchanger from Pharmacia (Piscataway, NJ.). SDS-PAGE molecular weight standards

were from Bio-Rad (Richmond, CA). DNA-cellulose was prepared according to Alberts & Herrick (1971). Benzamide-Sepharose 4B affinity matrix and bovine ADPRT enzyme protein were prepared as described earlier (Buki et al., 1987). Polyclonal antibody was raised in rabbits against the purified enzyme (Ferro et al., 1978). Coenzymic DNA was obtained as a side product from the ADPRT purification (Buki et al., 1987) and was rendered protein-free by phenol extraction. It consisted mainly of double-stranded DNA of 300 bp on average (Yoshihara et al., 1978, Niedergang et al., 1979). All other chemicals were of analytical grade.

Buffers. Buffer I (for digestion with plasmin): 50 mM HEPES, 150 mM NaCl, pH 8.0. Buffer II (for affinity chromatographies): 50 mM Tris, 200 mM NaCl, 50 mM sodium bisulfite, 10 mM 2-mercaptoethanol, 1 mM EDTA and 15% ethylene glycol, pH 7.3.

Methods. ADPRT enzyme activity was assayed as described (Buki et al., 1987). Polypeptides were separated according to Laemmli (1970) in 10% SDS-polyacrylamide gels (SDS-PAGE) and ADP-ribosylated polypeptides by an acid-urea gel electrophorectic method (Jackowski & Kun, 1983). High performance liquid chromatography of polypeptides was carried out with Beckman-Altex 100A pumps, gradient controller 421 and a variable wavelength detector. The amino acid composition of polypeptides was determined by the "picotag" method (Henrikson & Meredith, 1984) in a Waters 440 system and sequenced in a Model 470-A gas phase sequencer with an on-line 120A PTH-analyzer (Applied Biosystems, Foster City, CA.) according to Hunkapiller, et al. (1983).

The polypeptides in SDS-PAGE were stained with Coomassie Brillant Blue R 250 (0.1% in 50% aqueous ethanol/10% acetic acid) after brief fixation in 10% trichloroacetic acid. The protein content of electrophoretically separated bands was determined by extraction of the protein-bound Coomassie blue from the gel slices with 1 mL of 0.2M Tris base containing 40% ethanol, followed by spectrophotometry (λ = 595 nm) and the protein concentration was estimated from a calibration curve obtained from the same gel with known amounts (0 to 5 µg) of BSA. Alternatively polypeptides were electro-transferred onto nitrocellulose membranes in 25mM 3-(cyclohexylamino)-2-hydroxy-1-propane sulfonate buffer (pH 9.5) containing 20% methanol (Szewczyk & Kozloff, 1985) for 1.5 hours at 100V in a BioRad Trans-Blot Cell and immunostained as follows. Nitrocellulose membranes were first soaked in 1% BSA dissolved in PBS for one hour (blocking) and then washed with a solution containing 0.05% Tween 20, 0.01% Thimerosal in PBS (washing buffer, Engwall & Pearlman, 1971), 20 - 50 mL/plate. This step was followed by treatment with a solution of the specific antibody (1:5000 dilution in PBS) by incubation with gentle shaking for 1 hour at room temperature. The membranes were washed three times with the washing buffer (see above), then the peroxydase-labelled antirabbit goat serum (1:1000 dilution) was added and incubation continued at room temperature for another hour. After five washes with the washing buffer, the membranes were placed into a freshly prepared solution of 3,3'-diaminobenzidine (1 mg/mL in 30mM Tris-HCl, 150 mM NaCl, pH 7.3) and 1 µL of 30% H_2O_2 per mL of solution

was added, rapidly mixed and carefully shaken manually (1-5 min) until brown bands developed. The reaction was stopped by acidification with 6N HCl to bring the pH to 1.0. The membranes were then rinsed with water and dried.

Results

Digestion of ADPRT with plasmin. Incubation of ADPRT (2 mg/mL, in Buffer I) with 33 μg/mL plasmin at 25°C either in the absence or presence of coenzymic DNA (Yoshihara et al., 1978, Niedergang et al., 1979) resulted in complete disappearence of ADPRT activity following first order kinetics (not shown). When the digestion of ADPRT by plasmin was arrested with soybean trypsin inhibitor (20 μg per mL of digestion mixture) at various time intervals, and the quantity of the remaining intact ADPRT protein was determined from the corresponding SDS-PAGE bands (see Methods), inactivation and the rate of disappearance of the ADPRT molecule (M_r 120,000) ran exactly parallel. These results demonstrated that the enzymatic activity depended on the quantity of intact ADPRT molecules, and that the component peptides have no residual activity. Fig. 1 shows a typical course of digestion. Practically, no intact enzyme is seen at 10 minutes of digestion and only four polypeptide bands remain after 30 minutes: M_r 29,000, 36,000, 42,000 and 56,000. On some gels, the M_r 56,000 polypeptide separated into three closely spaced bands. This microheterogeneity of the M_r 56,000 peptide explains that even though the amino termini were free, no meaningful sequence analyses were obtained. Incubation of the polypeptide M_r 56,000

with plasmin, at a peptide/plasmin ratio of 10, for 20 minutes resulted in slow formation of the M_r 42,000 polypeptide and traces of M_r 14-18,000 peptides, proving a precursor - product relationship between the former two. The polypeptide of M_r42,000 was a doublet (see Fig. 4, lane 2) with identical amino termini and was partially sequenced (see below). The rate of cleavage of the polypeptide M_r 56,000 by plasmin was significantly accelerated by the presence of 1 mM 3-methoxy- benzamide.

Binding properties of polypeptides to affinity columns. The benzamide-sepharose 4B affinity matrix that recognizes NAD^+ binding sites (Burtscher et al., 1986; Buki et al., 1987) strongly bound polypeptide of M_r 56,000 and only partly bound the polypeptide of M_r 42,000, which also appeared in the flow-through fraction along with the nonadsorbed basic polypeptides (Fig. 2).Polypeptides retained on the benzamide affinity column were eluted with 1 mM 3-methoxybenzamide. Based on these observations, we conclude that polypeptide 56,000 has higher affinity to the benzamide column than plypeptide 42,000. On the other hand, the DNA cellulose column retained the basic polypeptides M_r 29,000 and 36,000, but not the 56,000 and 42,000 species (Fig 3). Elution of basic polypeptides from the DNA-cellulose column was carried out with a limited volume (3 mL) of 1M NaCl, which left traces of M_r 56,000 and 43,000 polypeptides nonspecifically adsorbed (lane 3, Fig. 3). However, more extensive washing with the low salt buffer prior to the application of 1M NaCl readily removed these non-specifically adsorbed polypeptides. Reactive Red 120 is believed to

recognize NAD^+ - or $NADP^+$-binding sites (Watson et al.,1978). Since we used this dye for purification of ADPRT (Buki et al., 1987), it was of interest to determine which polypeptide moiety would bind to it. The Reactive Red 120-agarose column completely bound from Buffer II all of polypeptides except for M_r 42,000 which it bound only partially. The bound polypeptides had differential affinities to the dye-matrix as disclosed by stepwise elution with increasing salt (Fig. 4).

Immunological properties of polypeptides. All polypeptides isolated after plasmin digestion reacted with the rabbit antiserum raised against ADPRT as shown by an immunotransblot of polypeptides separated by SDS-PAGE (Fig. 5), where lanes 3 and 4 illustrate the immunoreactivity of ADPRT and of plasmin-generated polypeptides. Since the immunoassay is at least 10 times more sensitive than the Coomassie stain, minor bands gave strong signals.

Large scale isolation of polypeptides, their amino acid analyses and partial sequences. One-half mg of ADPRT protein was digested with plasmmin for 30 minutes, as described above (legend to Fig 1), and the resulting polypeptides were applied to a benzamide-Sepharose 4B affinity column (1 mL bed vol.). The column was washed with 3 mL of Buffer II. The wash contained the nonadsorbed polypeptides (see Fig. 2). The bound polypeptides were then eluted with 3 mL of the same buffer, but containing 1 mM 3-methoxybenzamide. Both the flow-through and eluate fractions were concentrated to about 100 µL in Centricon 10 (Amicon, Danvers, MA.) ultrafiltration units. Peptides present in

the concentrated flow-through fraction were further separated on a MonoS HR 5/5 cation exchanger column by a salt gradient (legend to Fig. 6) and the three major peaks were collected and concentrated. Peaks A and B (in Fig. 6) exhibited the same mass (central inset), corresponding to M_r 36,000, but peak C was not homogeneous and had to be repurified. After concentration, this fraction was injected into the same MonoS HR 5/5 cation exchanger column and developed by the same salt gradient yielding one main peptide (M_r 29,000) and a second polypeptide of M_r 31,000 amounting to about 10% of the former. The proportion of these two polypeptide bands cannot be visually ascertained on the basis of the inset of Fig 6 because the gel was overloaded. The two polypeptides have tha same blocked N-terminus and therefore most probably represent slightly different chain lengths of the same region, owing to a small variation in the cutting site.

Separation of the polypeptides that were eluted with 3-methoxy-benzamide from the benzamide-Sepharose 4B column (M_r 42,000 and 56,000) was accomplished on a Spherogel TSK-3000 SW size exclusion column by recycling.

The amino acid composition of isolated polypeptides is shown in the Table. The DNA-binding polypeptides contain a high amount of lysine, especially the polypeptide of M_r 36.000 A (16.3%), and the lysine content of its sequenced N-terminal segment was even higher (23.5%, see below).

Out of the 5 analyzed polypeptides (Table) four had free amino termini. The polypeptide of M_r 29,000 was found to have a blocked

amino terminus, indicating that this polypeptide is the probable N-terminal fragment of ADPRT. The nature of the blocking group is unknown, except its identity with pyroglutamic acid was ruled out by its insensitivity to pyroglutaminase (not shown). The polypeptide of M_r 56,000 yielded no interpretable N-terminal sequence, because several PTH-amino acid signals were seen in each cycle, in agreement with the microheterogeneity observed on SDS-PAGEs.

The polypeptide of M_r 42,000, though always a doublet on SDS-PAGE, (lane 2 on fig. 4) had a uniform amino terminus and was sequenced to 32 cycles, yielding the following sequence:

(?)LTVNPGTKSKLPKPVQNLIKMIFDVESMK(K)A

(Parentheses indicate uncertainities of amino acids. Single letter codes are used according to IUPAC-IUB, 1986).

Sequencing of the polypeptides of M_r 36,000 A and B was carried out to 51 cycles. The polypeptide 36,000 A was found to be one lysine residue longer than B. The N-terminal sequence of the polypeptide 36,000 A is:

KSKKEKDKETKLEKALKAQNDLIWNVKDELKKA(C)STNDLKE(L)(L)IFNKQEVP.

The difference of one lysine between polypeptides M_r 36,000 A and B is corroborated by data for amino acid compositions (Table). For peptides of M_r 36,000 (300-310 amino acid residues) a difference of 0.4 mol % in lysine content corresponds to a difference of 1.2 mol of lysine.

ADP-ribosylation of ADPRT and of polypeptides generated by digestion with plasmin. The gel electrophoretic identification

of poly(ADP-ribosyl)ated peptides is uncertain even in special gel systems that preserve the base sensitive ADP-ribose-protein bonds (Jackowski & Kun, 1983), because long $(ADP\text{-}ribose)_n$ side-chains cause slower migration and comparison with standard polypeptides may be unreliable. It is for these reasons that we confined our experiments to mono-ADP-ribosylation, which is catalyzed by ADPRT at 100 nM NAD^+ (Kirsten et al., 1985; Bauer et al., 1986). Under these conditions mono-ADP-ribosylation of ADPRT stops when 50% of the enzyme molecules are mono-ADP-ribosylated (Hakam, A., McLick, J. & Kun, E., unpublished results).

When the plasmin digest was ADP-ribosylated with 100 nM [^{32}P]NAD^+ by a trace amount of ADPRT (see legend of Fig. 7A), the main labelled macromolecule was the intact ADPRT itself and of the polypeptides only the two basic ones (M_r 29,000 and M_r 36,000) were labelled and then only lightly as shown on Fig. 7A.

A markedly different picture was seen when ADPRT was first auto-[^{32}P]-ADP-ribosylated then digested with plasmin and the ensuing ADP-ribosylated polypeptides were separated by electrophoresis as above. All of the polypeptides were labelled, most of the label being on the M_r 36,000 and 56,000 species. In Fig 7B the arrows point to strongly labelled but faintly stained bands which were probably produced by alternative hydrolytic sites of plasmin due to ADP-ribosylation of ADPRT.

Discussion

The combined presence of the four stable polypeptide degradation

products generated by plasmin does not retain residual enzymatic activity, in contrast to the combined polypeptides obtained by limited proteolysis by papain (Kameshita et al., 1986) It may be assumed that plasmin, in contrast to papain, cuts the ADPRT molecule at or close to the catalytic site(s). Since digestion of ADPRT by plasmin coincides with a loss of some polypeptides, by the cleavage of the polypeptide M_r 56,000 to 42,000, inactivation of ADPRT by plasmin seems plausible. The significance of the site of peptidase action in determining residual enzymatic activity was demonstrated also by Kameshita et al. (1986) who found that limited hydrolysis by chymotrypsin eliminated enzymatic activity. We have previously estimated the distance between DNA- and NAD^+-binding sites on ADPRT to be around 8.5 Å (McLick et al., 1987), which would predict that catalytic site(s) may be in the vicinity of the DNA- and NAD^+ - binding polypeptides. However, the localization of catalytic site(s) must take into account the recently observed high degree of helicity of ADPRT (Buki, K.G., Yang, H.E., Kubota, S. & Kun, E. unpublished results), implying that a catalytic interaction of NAD^+- and DNA-binding site(s) may not reflect distances measured on linear polypeptide chains, but could contain significant structural factors characteristic for the ADPRT molecule. The enhancment of the plasmin-catalyzed cleavage of the polypeptide M_r 56,000 to M_r 42,000 by 3-methoxybenzamide supports the possibility that the binding of this nicotinamide analog to the polypeptide M_r 56,000 may induce conformational changes in the polypeptide favoring a proteolytic attack by

plasmin. Apparently allosteric kinetics of ADPRT (Bauer et al., 1986) further implies a correlation between catalytic and conformational properties of ADPRT.

Identification of the ligand binding properties of polypeptides generated from ADPRT by plasmin depended on affinity chromatography columns, which by their DNA and NAD^+ specificity complemented each other. The benzamide-Sepharose column proved more efficient for preparative purposes than the DNA-cellulose matrix because of higher stability and capacity. We have previously obtained kinetic evidence (Buki et al., 1987) indicating that Reactive Red 120 dye exhibited varying degrees of affinities towards both DNA- and NAD^+ -sites, which is also demonstrated by the results shown in Fig. 4.

According to amino acid composition and partial sequencing of the polypeptides, in addition to their behaviour on affinity columns, tentative arrangement of the polypeptides in the ADPRT structure can be proposed (Fig. 8). A blocked amino terminus is present in the polypeptide M_r 29,000, and therefore it is feasible that this is the amino terminus of ADPRT. According to Kameshita et al. (1984), the DNA-binding polypeptide domain of ADPRT is represented by a polypeptide of M_r 66,000, obtained by limited digestion with α-chymotrypsin. The two DNA-binding basic polypeptides (M_r 29,000 and 36,000), isolated from plasmin digests, are most likely components of this larger DNA-binding polypeptide and therefore are contiguous. The polypeptide of M_r 56,000 accounts for the rest of the ADPRT moloecule. This NAD^+-binding polypeptide triplet can be cleaved by plasmin to the M_r 42,000

doublet and a small peptide of M_r 14-18,000. This latter small polypeptide may contribute to the stronger binding of M_r 56,000 to benzamide-Sepharose matrix than M_r 42,000. The cleavage of the M_r 56,000 polypeptide triplet to the M_r 42,000 doublet yields the sequenceable amino terminus in the latter, and therefore its positioning as shown in Fig. 8 is probable.

Since digestion by plasmin caused no appreciable loss of protein-bound ADP-ribose (not shown), and the staining pattern of ADP-ribosylated polypeptides was indistinguishable from polypeptides obtained from the native ADPRT, artefacts due to the hydrolysis of ADP-ribose-protein bonds can be excluded. On the other hand Kameshita et al. (1984) digested the ADP-ribosylated enzyme sequentially with α-chymotrypsin and with papain at pH 8.0 and found that only the median polypeptide of M_r 22,000 became ADP-ribosylated. It cannot be ruled out that repeated exposure of alkali-labile ADP-ribosyl-protein bonds to pH 8.0 caused a loss of label (Bauer et al., 1986), thus only the most stable or abundant ADP-ribose-protein adducts were experimentally identified. Holtlund et al. (1983) reported that both ADPRT and two of its isolated endogenous degradation products (M_r 59,000 and M_r 72,000) were ADP-ribosylated. Furthermore, most of the cyanogen bromide-generated fragments contained ADP-ribose, and since they were not exposed to base, a pattern of auto-ADP-ribosylation was obtained that was similar to ours.

The enzymatic ADP-ribosylation of basic polypeptides (Fig. 7A) may be explained by their DNA binding. Similarly one may

postulate that ADP-ribosylation of proteins other than ADPRT always requires their binding to DNA.

Acknowledgement

We are indebted to Dr. Andras Patthy (Biomolecular Resource Center) for amino acid analysis and sequencing.

Table

Amino acid composition of isolated polypeptides obtained from digestion with plasmin [a]

Amino acids	M_r of polypeptide				
	29,000	36,000 A	36,000 B	42,000	56,000
Asx	6.6	8.8	9.5	11.0	6.8
Glx	9.1	12.2	12.9	10.8	10.2
Ser	9.2	7.6	8.0	7.9	10.9
Gly	7.8	5.1	5.5	7.7	14.7
His	1.8	0.6	0.7	2.5	1.7
Arg	4.6	2.5	2.4	3.4	3.8
Thr	4.2	5.1	5.2	4.5	3.3
Ala	5.5	8.6	8.3	5.3	11.5
Pro	4.4	6.4	6.1	4.7	5.0
Tyr	4.9	0.1	0.1	4.3	5.0
Val	6.4	7.3	6.9	6.3	5.0
Met	5.1	2.8	2.7	3.3	1.7
Cys	1.6	0.3	0.5	0.3	3.1
Ile	5.4	4.7	4.7	5.1	2.5
Leu	4.8	8.6	8.5	8.7	6.7
Phe	5.4	2.9	2.7	3.1	3.0
Lys	13.3	16.4	16.0	10.9	5.0

Legends to Figures

Fig.1. SDS-PAGE analysis of digestion products of ADPRT with plasmin.

Digestion was carried out as described in Results. At indicated time points, aliquots were withdrawn, mixed with an equal volume of SDS-PAGE sample buffer containing 5 mM PMSF, and heated to 90°C for one minute. Each lane represents a total of 4 μg of protein, and staining is with Coomassie blue. Molecular weight standards (as $M_r x 10^3$) are shown along the left side of the gel: β-galactosidase, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysosyme (BioRad sdtandards). The molecular weights of polypeptides are indicated on the right side. All other figures of gels contain the same notation.

Fig. 2. SDS-PAGE analysis of polypeptides obtained by benzamide-Sepharose chromatography

After digestion with plasmin as described in Results, 200 μg of ADPRT-digest was diluted with an equal volume of Buffer II, containing also 5 mM PMSF, and the solution was applied onto a benzamide-Sepharose column of 0.5 mL volume. The column was washed with 2 mL of Buffer II and then eluted with 2 mL of 1 mM 3-methoxybenzamide dissolved in the same buffer. The wash and the eluate were concentrated in Centricon 10 microconcentrators and applied to SDS-PAGE (5 μg of protein per lane). Lane 1: unseparated plasmin digest; lane 2: flow-through fraction; lane 3: polypeptides eluted with 3-methoxybenzamide.

Fig.3. SDS-PAGE analysis of polypeptides separated by DNA-cellulose

chromatography

As described in the legend to Fig. 2, 100 μg of plasmin digest was prepared and applied to a DNA-cellulose column of 1 mL volume. The column was washed with 3 mL of Buffer II and then eluted with the same buffer containing 1.0 M NaCl. The wash and the eluate were concentrated in Centricon 10 microconcentrators and applied to SDS-PAGE (3 μg of protein per lane). Lane 1: unseparated plasmin digest; lane 2: flow-through fraction; lane 3: polypeptides eluted with high salt.

Fig. 4. SDS-PAGE analysis of polypeptides separated by chromatography on Reactive Red 120-agarose

ADPRT (200 μg) was digested with plasmin, as described in Results, for 10 minutes. The digestion was stopped as described in the legend to Fig 2. The peptide mixture was applied onto a Reactive Red 120-agarose column of 0.5 mL volume and eluted stepwise with 1 mL portions of Buffer II containing increasing concentrations of NaCl. Lane 1: unseparated plasmin digest; lane 2: wash fraction with Buffer II; lanes 3,4 and 5: eluates with 0.4, 0.6 and 0.8 M NaCl in Buffer II respectively.

Fig. 5. Electroblotting and immunostaining of ADPRT and its component peptides

A plasmin digest of ADPRT, as described in the legend of Fig 1 (30 min), was subjected in duplicate to SDS-PAGE as described in legend to Fig 1, with undigested ADPRT as control. The developed gel was cut lengthwise and one half was stained with Coomassie blue and the other was transblotted and immunostained as described in Methods. Lanes 1 and 2: Coomassie-stained ADPRT and

its digest, respectively; lanes 3 and 4: immunostained transblots of the same.

Fig. 6. Separation of basic polypeptides on MonoS HR 5/5 cation-exchanger column

A MonoS HR 5/5 column was fitted with HPLC components described in Materials. Buffer A was 25 mM HEPES, 50 mM NaCl, 10 mM 2-mercaptoethanol, pH 7.7; Buffer B was the same buffer with 1.0 M NaCl. The mixture of basic peptides (approx. 200 µg) was equilibrated with Buffer A in a Centricon 10 microconcentrator and injected into the column. The flow rate was 1 mL/min. After 4 min of isocratic flow with buffer A, a linear gradient from A to B (30 min) was started (indicated at top) and peak fractions were collected by hand (marked as ⊢⊣). Aliquots of the collected fractions A, B and C were analyzed by Coomassie blue-stained SDS-PAGE (inset).

Fig.7. SDS-PAGE analysis of auto[^{32}P]ADP-ribosylation products

A: ADP-ribosylation of a mixture of polypeptides obtained by digestion of 22 µg of ADPRT by plasmin was carried out by incubating it with 0.32 µg of ADPRT in the presence of 6 µg of coenzymic DNA in a 25 µL reaction mixture consisting of 50 mM Tris, 2,5 mM MgCl, 1 mM dithiothreitol, 0.1 µM [^{32}P]NAD^+ (10^6cpm) and 0.3 mM PMSF, pH 7. The reaction was stopped after incubation at 25°C for 1 min by addition of an equal volume of sample buffer of the acid urea SDS-PAGE system, and one third volume was applied onto the gel. Auto-ADP-ribosylation of ADPRT was carried out in the absence of polypeptides. Gels were run at room

temperature, stained with Coomassie blue, dried and exposed to X-ray films overnight. Lane 1: ADPRT alone; lane 2: ADP-ribosylated polypeptide digest (both lanes 1 and 2 are Coomassie-stained); lanes 3 and 4 are autoradiograms of lanes 1 and 2 respectively. B: ADPRT, 32 μg, was auto[^{32}P]ADP-ribosylated in the presence of 20 μg of coenzymic DNA in a 25 μL volume for 30 seconds, since rates of auto-ADP-ribosylation of ADPRT are very rapid. The rest of the system was the same as in A, except PMSF was omitted. The reaction was terminated with 2 μL of 100 mM ethanolic 3-methoxybenzamide and a 7 μL aliquot mixed with an equal volume of sample buffer and applied to acid urea SDS-PAGE. To the remainder, 0.7 μg of plasmin was added and digestion was allowed for 20 min at 25°C. Following the addition of an equal volume of sample buffer, the digest was subjected to electrophoresis along with the undigested sample. Visualization of gels was carried out as in A. Lane 1: undigested ADPRT; lane 2: ADPRT-derived polypeptides (both lanes Coomassie-stained); lanes 3 and 4 are autoradiographies of lanes 1 and 2 respectively.

Fig. 8. Proposed arrangement of isolated polypeptides and primary structure of ADPRT

The solid boxes refer to the sequenced region and the spikes indicate the frequency (not the location) of auto-ADP-ribosylation on the primary polypeptide structure.

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Fig 1

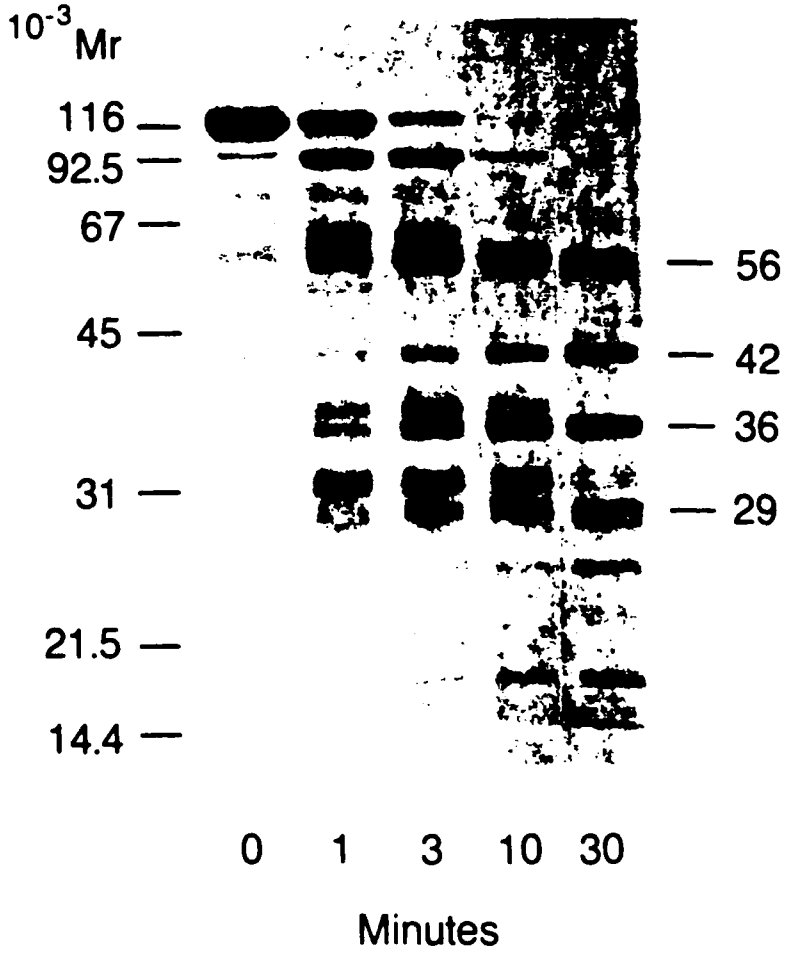
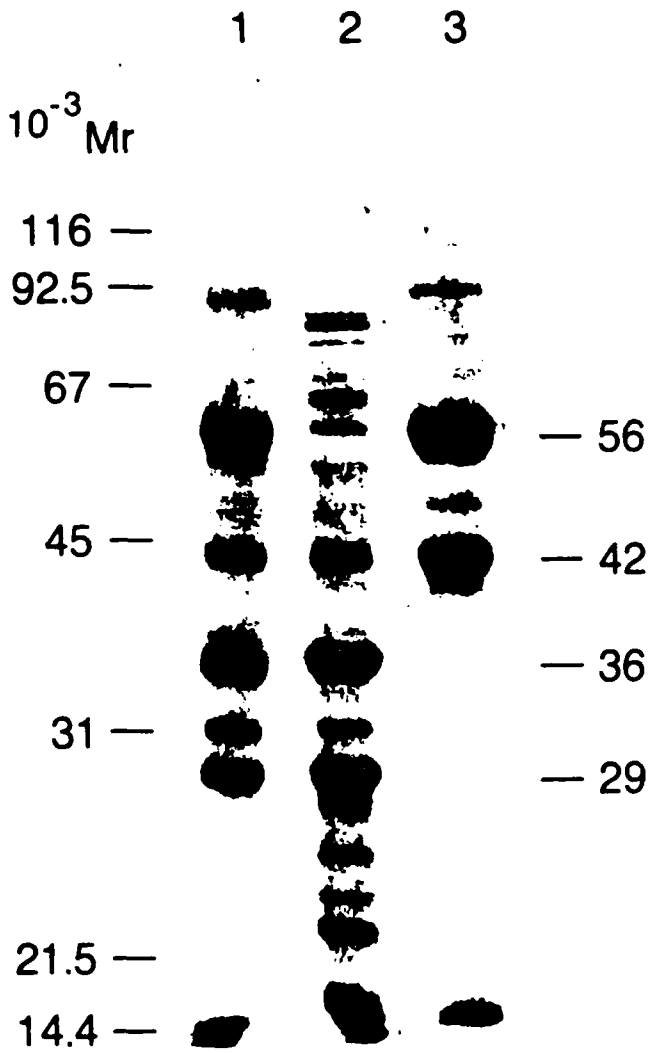


Fig 2



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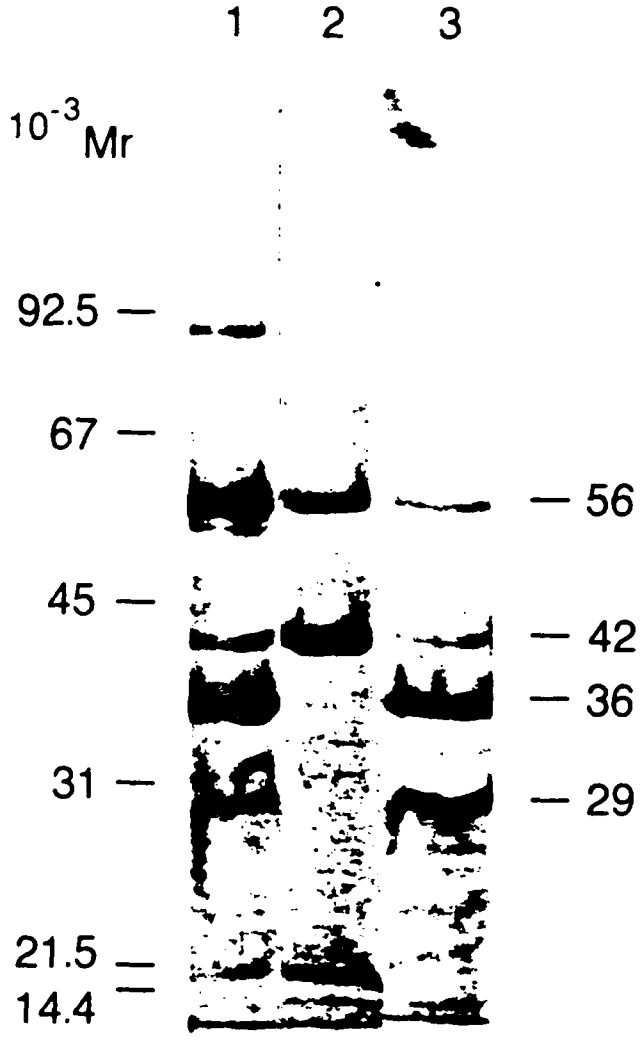

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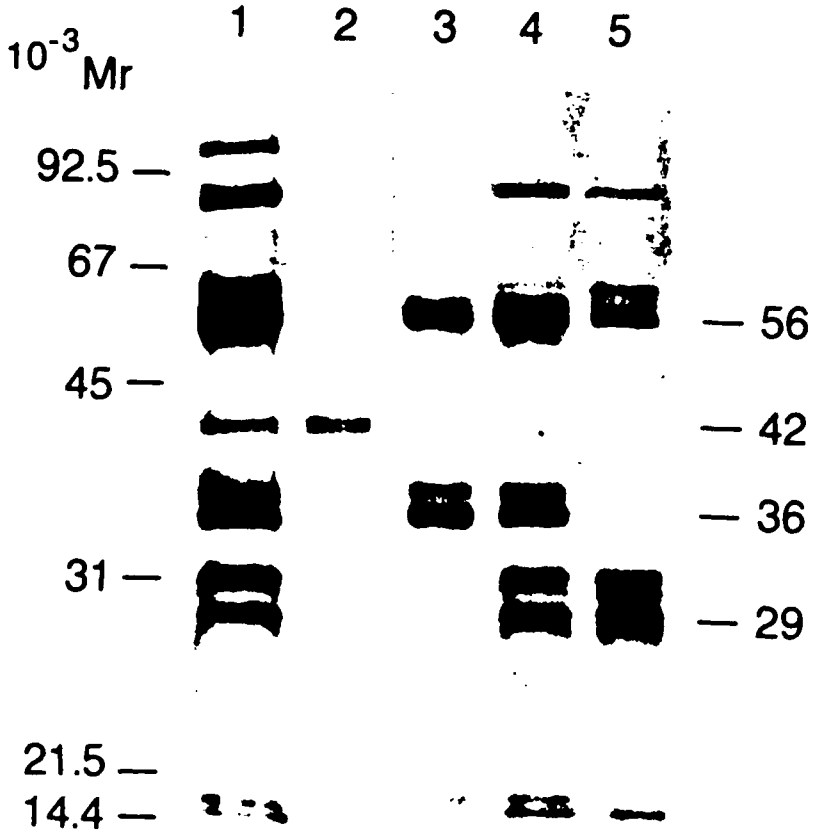

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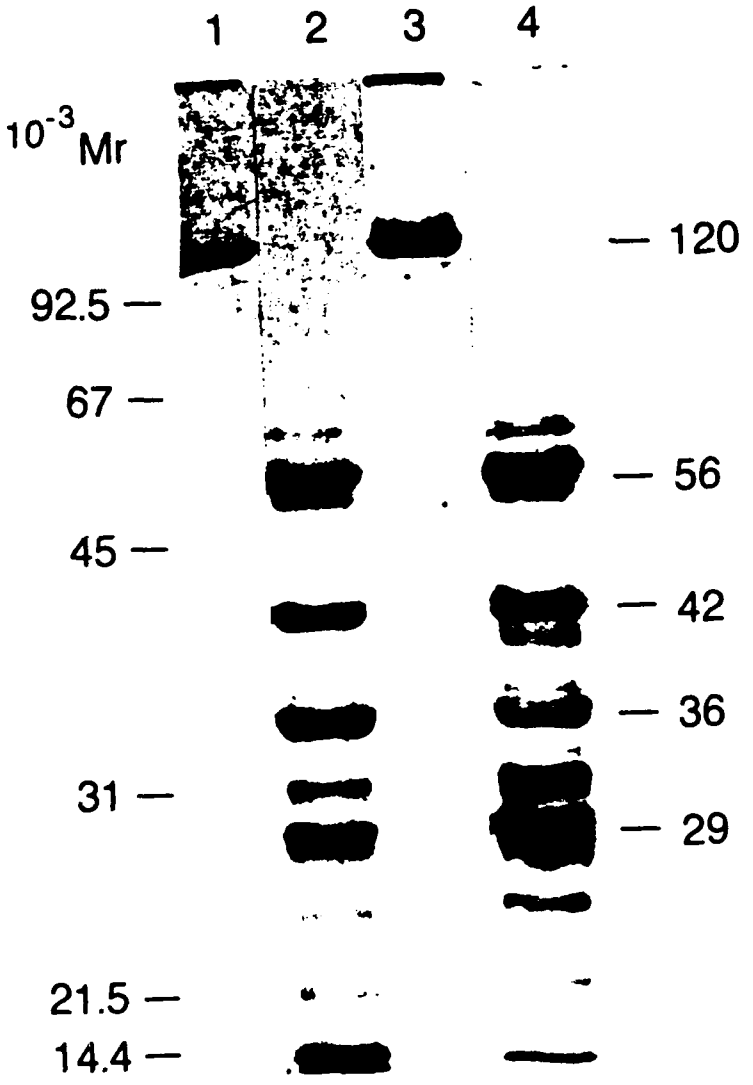

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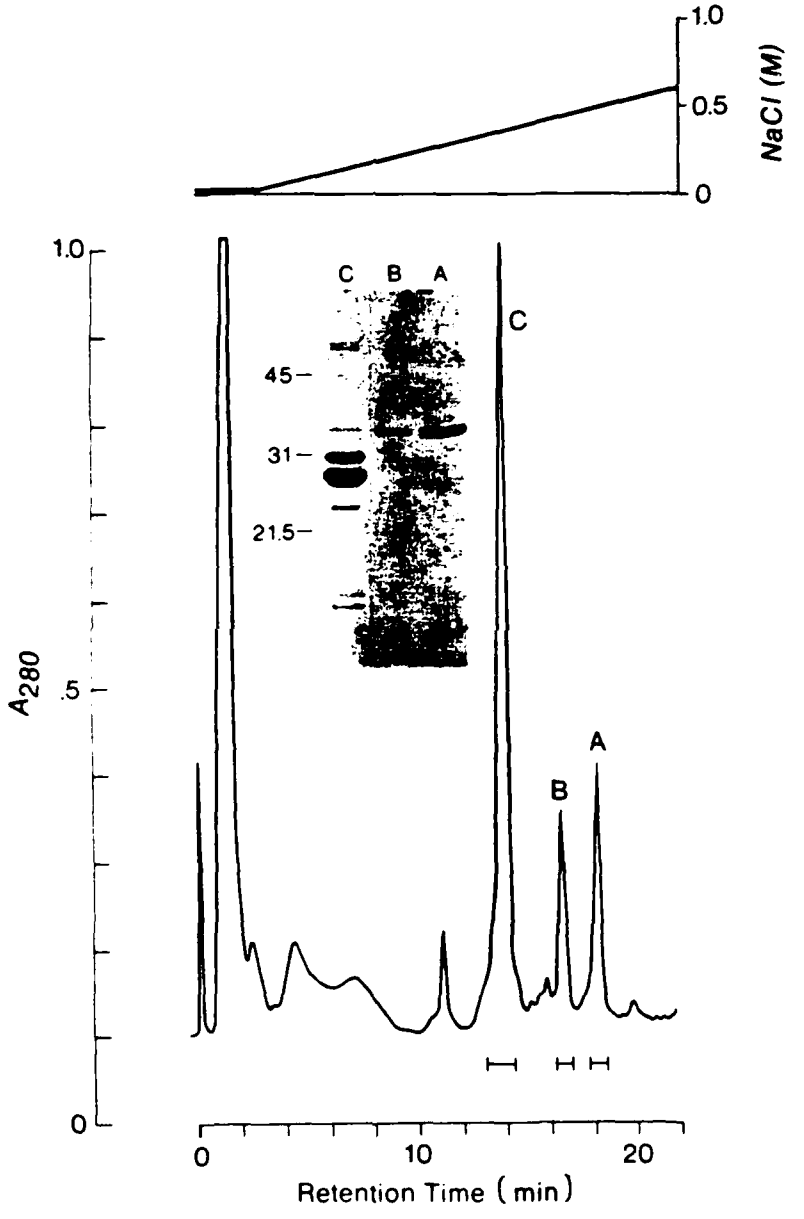
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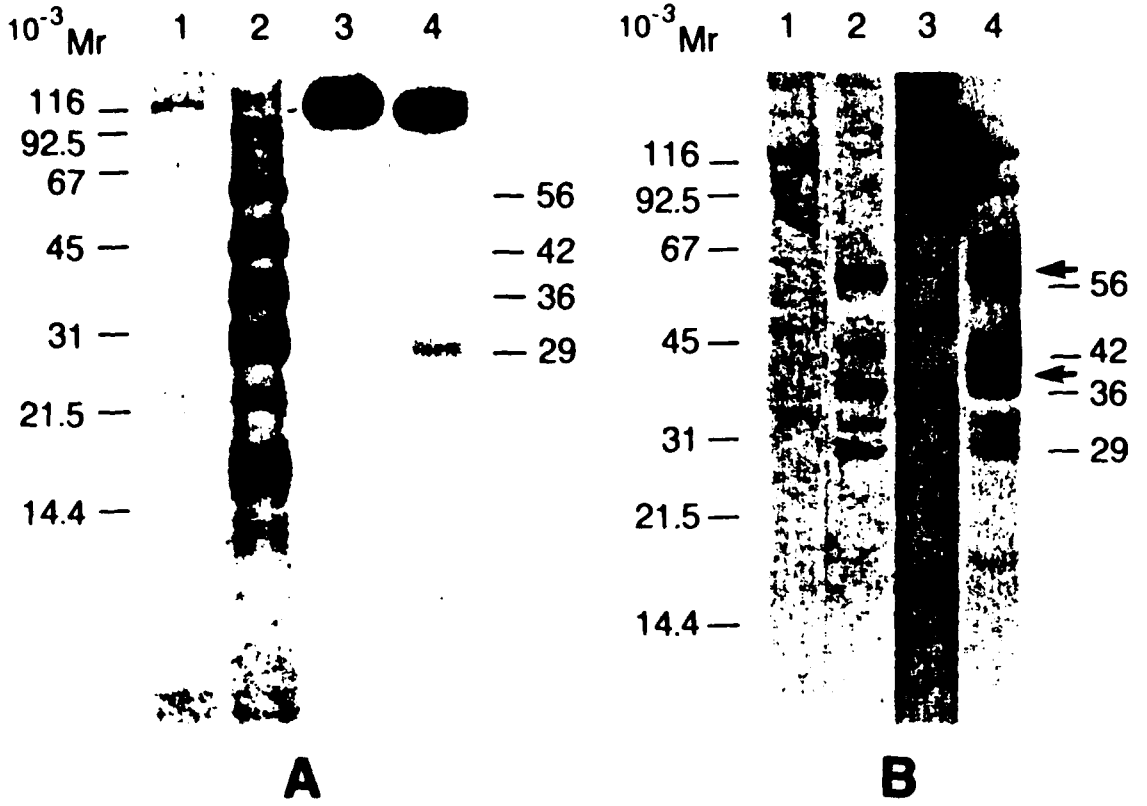
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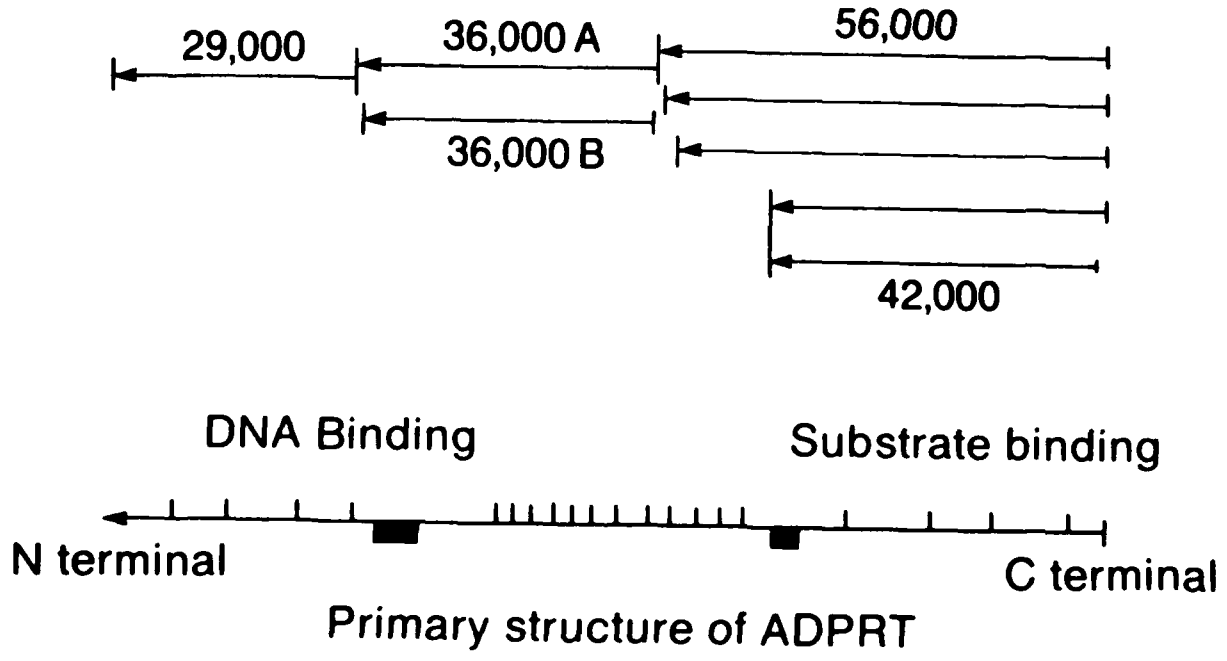
Fig 8



Primary structure of ADPRT

III

Molecular Cloning of the Bovine Poly(ADP-ribose) Polymerase Gene

A. Cloning Strategy:

We employed oligonucleotide probes to identify potential clones from a bovine liver cDNA lambda gt11 library. A bovine liver cDNA library was chosen because it has been reported earlier that the poly(ADP-ribose) content per unit weight of tissue was higher for liver than other tissues analyzed (1). Therefore, by inference it is possible that the polymerase enzyme and polymerase -specific mRNA content could be considerably higher in liver than in other tissues. A cDNA library would be a more appropriate source to screen for the polymerase gene than a genomic library. In addition, fewer cDNA plaques or colonies (10^6 - 10^7) can be screened than in the case of a genomic library (10^8 - 10^{10}). Recently, a procedure for the rapid purification of the poly(ADP-ribose) polymerase protein was devised in this laboratory (2). This greatly facilitated amino acid sequencing of peptide fragments of the bovine polymerase protein. The partial amino acid sequence of a 36 kD polypeptide (obtained by digestion of the enzyme with plasmin) was utilized in the design of the oligonucleotide probes. A partial mRNA and cDNA sequence was deduced from the amino acid sequence taking into consideration the codon utilization data of Chen et al.(3). Two types of oligonucleotide probes were synthesized in collaboration with Dr. Corey Levinson (Cetus Corp., Emeryville,CA).

Probes A: Only two of the most frequently utilized bases in the "wobble" position were considered for the design of these probes (Fig.1).

Probes B: Inosine in the "wobble" position of each codon; inosine can base-pair with all four bases.

All the above probes were 36 nucleotides long.

Bovine liver lambda gt 11 cDNA library was purchased from ClonTech Corp. (Palo Alto,CA). This library had approx. 1.6 x 10 independent clones with insert sizes ranging from 0.24 - 4 kb (as informed by the supplier).

b. Screening Procedure

E.coli y1090 was grown to late log phase in LB broth + 0.2% maltose. The bacteria were infected with the lambda gt11 cDNA library at a multiplicity of infection of approx. 0.7. The phage was plated with top-agarose (0.8%) so that there would be approximately 30-50,000 plaques per plate (150 mm diameter). The plates were incubated over-night at 37°C. After the plaques were approx. 1 mm in diameter the plates were chilled to 4°C for 2-3 hours. Nitrocellulose filter discs (130 mm diameter) were overlaid onto the plates and the phage adsorbed onto the filters. The phage on the filters was denatured in 1.5 M NaCl + 0.5 M NaOH for 1-2 min. and neutralized in 0.5 M Tris-Cl (pH 8.0)+ 1.5 M NaCl for 8 min. The filters were then washed in 3 X SSC for 5-10 min. and subsequently baked at 65°C overnight.

Filter-discs were pre-hybridized for 4 hrs. in 5 X Denhardt's Solution + 6 X SSC + 50 mM Na-phosphate, pH 6.8. at 22°C and hybridized in the same solution for 48 hours in the presence of

^{32}P-labelled mixture of probes A and B (section A). The washing protocol was as follows:

1 st wash: 6XSSC + 0.1% SDS for 30 min.

2nd wash: 3XSSC + 0.1% SDS for 30 min.

3rd wash: 1XSSC + 0.1% SDS for 30 min.

4th wash: 0.11XSSC + 0.1% SDS for 15 min.

After these washes the filters were dried at 65°C and exposed to X-ray film over-night with an intensifying screen. All plates were screened in duplicates with appropriate markings. Duplicate signals were identified and a region around the signals was picked from the phage plates. These isolates were re-screened in the same way as described above. In the primary screening approx 2 x 10^6 plaques were screened and in the secondary screening 20,000 plaques per primary isolate. In the secondary screening 50 individual duplicate positive plaques were picked. Mini-DNA preparations were made from these 50 isolates and re-screened with ^{32}P-oligonucleotide probes in a dot-hybridization matrix with lambda DNA and beta-Actin-DNA serving as controls. Two strongly positive clones, tentatively designated pSKK 13 and pSKK 34 were identified. Restriction endonuclease digestion of pSKK 13 and pSKK 34 DNAs with EcoR I revealed that both the clones had DNA inserts of about 5-6 kb (Fig.2). It is important to note that the cDNA of pSKK13 and pSKK34 could probably be derived from a full-length (processed or unprocessed) mRNA transcript of the poly (ADP-ribose) polymerase gene since the cDNA insert is sufficiently long to be translated into a full-length polymerase protein.

C. Conclusions and Experiments in Progress.

We have isolated two lambda gt11 clones which presumably are derived from the poly(ADP-ribose) polymerase gene. To confirm that these are indeed clones containing inserts of this gene we are currently subjecting the two clones to the following tests.

1. Immunological identification of the polymerase epitopes in pSKK13 and/or pSKK34 lysates.

Crude lysates from pSKK13 and pSKK34 willbe prepared according to the method of Huynh et al. (4). A polyclonal antibody which is available in our laboratory will be used to detect the beta-galactosidase-poly(ADP-ribose) polymerase fusion peptide by Western blotting. The identification of an antigenically reactive fusion peptide could confirm the identity of either one or both of the lambda gt11 clones as containing parts of the polymerase gene.

2. Hybrid-selected or hybrid-arrested translation.

The insert DNA will be excised from pSKK13 and pSKK34, immobilized onto nitrocellulose filters, and mRNA in the size range of 5 kb will be selected by binding total poly-A mRNA to the insert DNA. This selected RNA, exspected to be in the 5-6 kb range, will be translated in reticulocyte or wheat germ extract translational system and a polyclonal antibody will be employed to detect the complete or partially synthesized polymerase protein by Western blotting. Hybrid-arrested translation - wherein the cDNA insert- mediated arrest of antigenically -reactive polymerase synthesis could be monitored, can also be employed to

confirm the identity of pSKK13 and pSKK34.

Once the identity of either one or both of the clones pSKK13 and pSKK34 as poly(ADP-ribose) polymerase clones is established, the following experiments will be undertaken.

a. Isolation of a genomic clone for poly(ADP-ribose) polymerase:

A bovine genomic library (EMBL-3, appr. 15 kb insert size) will be screened with either one of the two P-labelled inserts which are determined to be clones for poly(ADP-ribose) polymerase in the previous experiments. A full-length clone of the poly(ADP-ribose) polymerase gene will then be subjected to restriction endonuclease mapping.

b. DNA sequencing of the cDNA inserts:

The isolated cDNA inserts will be subcloned into phage M13 vectors or pGEM vectors (Promega Labs., Madison, Wis.) and the DNA sequence will be determined by the Sanger method of sequencing.

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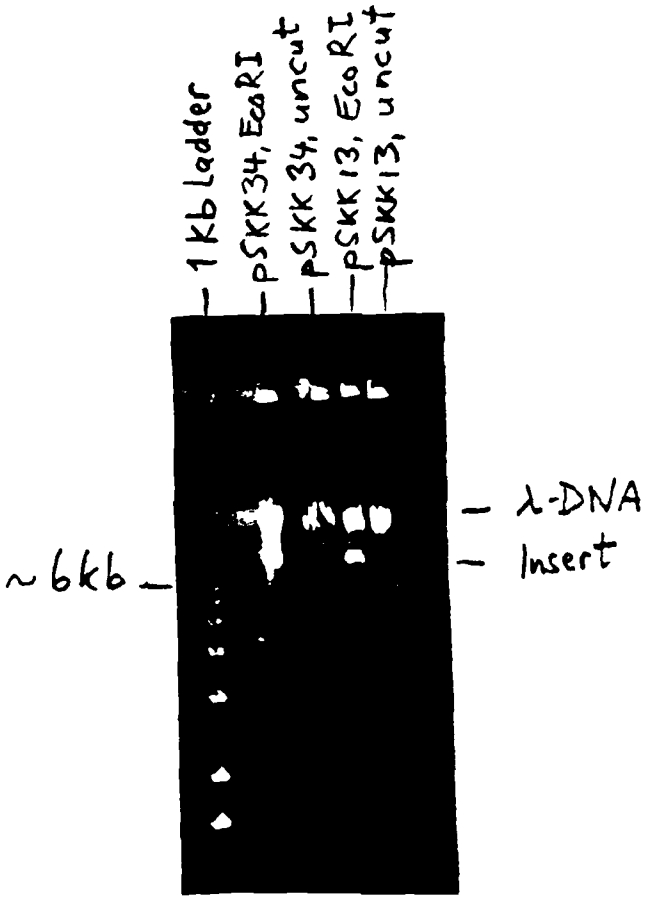
Figure 1

Protein Sequence											
NH_2- leu	ile	lys	met	ile	phe	asp	val	glu	ser	met	lys- COOH
mRNA											
5'-CU(G/C)	AU(U/C)	AA(A/G)	AUG	AU(U/C)	UU(U/C)	GA(U/C)	GU(G/C)	GA(G/A)	UC(U/C) AGC	AUG	AA(A/G) -3'
cDNA											
3'-GA(C/G)	TA(A/G)	TT(T/C)	TAC	TA(A/G)	AA(A/G)	CT(A/G)	CA(C/G)	CT(C/T)	AG(A/G) TCG	TAC	TT(T/C) -5'
5'-CT(G/C)	AT(T/C)	AA(A/G)	ATG	AT(T/C)	TT(T/C)	GA(T/C)	GT(G/C)	GA(G/A)	TC(T/C) AGC	ATG	AA(A/G)-3'

Amino acid - and predicted nucleotide sequence of the oligonucleotide probes.

Figure 2



Ethidium bromide - stained 1 % agarose gel, containing DNA from clones pSKK13 and pSKK34, before and after digestion with EcoR I.

END

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